In vivo and *in vitro* maturation of rabbit oocytes differently affects the gene expression profile, mitochondrial distribution, apoptosis and early embryo development

M. Arias-Á lvarez[E], *R. M. García-García, J. López-Tello, P. G. Rebollar, A. Gutiérrez-Adán and P. L. Lorenzo*

Abstract. *In vivo*-matured cumulus–oocyte complexes are valuable models in which to assess potential biomarkers of rabbit oocyte quality that contribute to enhanced IVM systems. In the present study we compared some gene markers of oocytes and cumulus cells (CCs) from immature, *in vivo*-matured and IVM oocytes. Moreover, apoptosis in CCs, nuclear maturation, mitochondrial reallocation and the developmental potential of oocytes after IVF were assessed. In relation to cumulus expansion, gene expression of gap junction protein, alpha 1, 43 kDa (*Gja1*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*) was significantly lower in CCs after *in vivo* maturation than IVM. In addition, there were differences in gene expression after *in vivo* maturation versus IVM in both oocytes and CCs for genes related to cell cycle regulation and apoptosis (V-Akt murine thymoma viral oncogene homologue 1 (*Akt1*), tumour protein 53 (*Tp53*), caspase 3, apoptosis-related cysteine protease (*Casp3*)), oxidative response (superoxide dismutase 2, mitochondrial (*Sod2*)) and metabolism (glucose-6-phosphate dehydrogenase (*G6pd*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)). *In vivo*-matured CCs had a lower apoptosis rate than IVM and immature CCs. Meiotic progression, mitochondrial migration to the periphery and developmental competence were higher for *in vivo*-matured than IVM oocytes. In conclusion, differences in oocyte developmental capacity after IVM or *in vivo* maturation are accompanied by significant changes in transcript abundance in oocytes and their surrounding CCs, meiotic rate, mitochondrial distribution and apoptotic index. Some of the genes investigated, such as *Gja1*, could be potential biomarkers for oocyte developmental competence in the rabbit model, helping improve *in vitro* culture systems in these species.

Introduction

Oocyte maturation is a crucial process for species survival, during which oocytes acquire their intrinsic capacity to support the subsequent stages of development, namely fertilisation and early embryo development (Eppig *et al.* 1997). In rabbit, IVM is a useful tool in biotechnology to produce somatic cell nuclear transfer (SCNT) embryos, for animal breeding, for studies of developmental biology and as a model system for human reproduction (Fischer *et al.* 2012). Despite the importance of rabbits as laboratory animal models, the efficiency of IVM remains very low in this species compared with other species because of the limited information available regarding the cellular and molecular mechanisms controlling oocyte maturation in rabbits.

It is well known that the several layers of cumulus cells (CCs) surrounding the oocyte play an essential function in follicular development and oocyte cytoplasmic maturation (Buccione *et al.* 1990). Indeed, the oocyte plays a dominant role in regulating the functions of CCs during folliculogenesis and it is therefore believed that the quality of CCs indirectly reflect an oocyte's competence (Eppig *et al.* 1997). Consequently, gene expression analysis in CCs provides a non-invasive method for

identification of the most competent oocytes for assisted reproductive technologies (ARTs). In recent years, several studies in animal models (Nivet *et al.* 2013; Blaha *et al.* 2015; Shao *et al.* 2015) and humans (McKenzie *et al.* 2004; Adriaenssens *et al.* 2010; Assou *et al.* 2010; Li *et al.* 2015) have tried to identify candidate genes expressed in CCs that could be used as biomarkers of oocyte quality. In this sense, some studies have focused on identifying differences in gene expression between IVM and *in vivo*-matured cumulus–oocyte complexes (COCs) in many species, including dogs (Cho *et al.* 2016), cattle (Tesfaye *et al.* 2009; Adona *et al.* 2016), mice (Cecconi *et al.* 2010) and humans (Jones *et al.* 2008; Ouandaogo *et al.* 2012). The results from these studies show that the expression of transcripts in CCs and oocytes is altered by *in vitro* conditions, giving rise to developmentally incompetent oocytes. Therefore, *in vivo*-matured COCs are a good reference to identify biomarkers of high oocyte developmental competence in CCs to improve the IVM protocols in animals and humans. However, limited data are available regarding gene expression profiles in association with oocyte maturation and, in some cases, the genes proposed as biomarkers exhibit differences in expression between studies (Burnik Papler *et al.* 2015). To the best of our knowledge, there is no information regarding these markers in rabbit models in the literature.

The complex events that occur during mammalian oocyte maturation include a coordinated nuclear and cytoplasmic maturation process (Sirard 2001). Nuclear maturation can be easily assessed because it is characterised by meiotic resumption, accompanied by CC expansion, meiotic progression until MII and release of the first polar body. Thereby, gene expression profiles of genes related to cumulus expansion, such as gap junction protein, alpha 1, 43 kDa (*Gja1*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*), have been proposed as suitable biomarkers for predicting pregnancy (Feuerstein *et al.* 2007; Hasegawa *et al.* 2007; Li *et al.* 2015; Shao *et al.* 2015). However, molecular events and reorganisation of organelles that occur during cytoplasmic maturation are difficult to evaluate and deserve further investigation.

Conversely, mitochondrial relocation in the oocyte has been widely used in several species to estimate oocyte cytoplasmic maturation because meiotic progression and CC expansion are accompanied by changes in the distribution of active mitochondria (Bavister and Squirrell 2000). These organelles play an important role in ATP synthesis by oxidative phosphorylation and are involved in the antioxidant defence of the oocyte (Dumollard *et al.* 2009). *In vitro* culture conditions are associated with abnormal mitochondrial reorganisation and dysfunction and, thus, with the onset of oxidative stress (Lord *et al.* 2013). Indeed, excessive reactive oxygen species (ROS) induce cell cycle arrest and oocyte damage via apoptosis in CCs (Torner *et al.* 2004). Therefore, excess of ROS contribute to the reduced developmental competence in oocytes from humans (Wilding *et al.* 2001; for a review, see Van Blerkom 2011), mice (Thouas *et al.* 2004) and bovine (Stojkovic *et al.* 2001).

Taking into account these premises, the first aim of the present study was to compare the gene expression of *Gja1* and *Ptgs2* mRNA in CCs from *in vivo*-matured oocytes (with presumably higher developmental competence) compared with IVM and immature oocytes (with potentially lower developmental competence). In addition, we analysed the expression of other putative transcripts involved in cell cycle and viability (V-Akt murine thymoma viral oncogene homologue 1 (*Akt1*)), cell cycle regulation and apoptosis (tumour protein 53 (*Tp53*), caspase 3, apoptosis-related cysteine protease (*Casp3*)), oxidative response (superoxide dismutase 2, mitochondrial (*Sod2*)) and metabolism (glucose-6-phosphate dehydrogenase (*G6pd*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)) in germinal vesicle (GV) immature, *in vivo*-matured and IVM oocytes and their CCs to identify potential target genes related to differences in the developmental competence of oocytes. Meiotic progression, cytoplasmic maturation (based on establishment of mitochondrial distribution patterns), early embryo development after IVF and the apoptotic rate in CCs were assessed to corroborate the genetic pool related to oocyte quality of rabbit COCs.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma Chemical.

Oocyte collection and IVM

Immature oocytes for IVM were recovered from adult New Zealand × California white rabbit does (*Oryctolagus cuniculus*; $n = 30$) located in the facilities of the Polytechnic University of Madrid. Ovaries were obtained by mid-ventral laparotomy of does after they had been sedated with 35 mg kg^{-1} ketamine (Imalgene1000; Merial) and killed using an intravenous bolus of barbiturate (30 mg kg^{-1}; Dolethal; Vetoquinol). The procedures were performed in accordance with the policies of the University Scientific Ethics Committee and the Spanish Policy for Animal Protection (RD53/2013), which meets the European Union Directive about the protection of animals used in experimentation.

Then, ovaries were placed in phosphate-buffered saline (PBS) at 37°C and transported to the laboratory. Ovarian follicles ≥1 mm in diameter were aspirated with a 2-mL syringe and 23-g needle under a stereoscopic microscope (Nikon). COCs with a compact cumulus were washed with PBS supplemented with 1 mg mL^{-1} polyvinylpolypyrrolidone (PVP) and selected on the basis of the morphological appearance of the CCs and cytoplasmic homogeneity. Immature COCs were processed for quantification of mRNA transcripts and for investigation of mitochondrial distribution. IVM COCs were obtained after culture for 16 h at 38°C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium consisted of tissue culture media (TCM)-199 with 2 mM L-glutamine, 0.1 mg mL^{-1} sodium pyruvate, 0.3% bovine serum albumin (BSA) and 10 ng mL^{-1} epidermal growth factor (EGF), as reported previously (Lorenzo *et al.* 1996).

To obtain *in vivo*-matured COCs, 27 does were killed, as described above, 15 h after the induction of ovulation. Ovulation was induced by intramuscular injection of gonadorelin, a synthetic analogue of gonadotrophin-releasing hormone (20 μg, i.m.; Inducel-GnRH; Ovejero). Ovulated oocytes were recovered by flushing the reproductive tract with PBS supplemented with 1 mg mL^{-1} PVP.

Table 1. Primers used for amplification of genes of interest from rabbit oocytes and cumulus cells using quantitative reverse transcription–polymerase chain reaction

H2afz, Histone family member z; *Gja1*, gap junction protein, alpha 1, 43 kDa; *Ptgs2*, prostaglandin-endoperoxide synthase 2; *Akt1*, V-Akt murine thymoma viral oncogene homologue 1; *Tp53*, tumour protein 53; *Casp3*, caspase 3, apoptosis-related cysteine protease; *Sod2*, superoxide dismutase 2, mitochondrial; *G6pd*, glucose-6-phosphate dehydrogenase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase

Gene	Primer sequence (5′–3′)	Fragment size (bp)	GenBank Accession no.
H2afz	Forward: AGGACGACTAGCCATGGACGTGTG Reverse: CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
Gja1	Forward: TGCCTTTCGTTGTAACACTCA Reverse: AGAACACATGAGCCAAGTACA	142	NM_001198948.1
Ptgs2	Forward: TCCAAGCTGGCCTCACTGATGG Reverse: AGCATGTGTGTGGCCCGACTTG	169	NM_001082388.1
Akt1	Forward: CACCACTGGATTTCTCTGCCT Reverse: GGTACCGTTTGTTGACTGTGC	230	XM_008257300.1
Tp53	Forward: GTGCTGACCAGGGACACGGC Reverse: CTGCACCAGGGCAGACCAGC	223	NM_001082404.1
Casp3	Forward: ACCCAGAAGACTGTGGATGG Reverse: AYGCCTGCTTCACCACCTTC	247	BC102589
Sod2	Forward: GCTTACAGATTGCTGCTTGT Reverse: AAGGTAATAAGCATGCTCCC	101	S67818.1
G6pd	Forward: CTGATCCTGGGTCGCTTCAT Reverse: ACGTACATGGGCACAAAACCA	68	NM_174602.2
Gapdh	Forward: CGCTGGGACGGGGTGCCCTTCATC Reverse: CGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4

RNA extraction and reverse transcription in COCs

In all, 132 COCs were used for mRNA abundance analysis ($n = 45$, 45 and 42 immature, IVM and *in vivo*-matured COCs respectively). CCs were mechanically removed by gentle repeated pipetting in PBS + 1 mg mL^{-1} PVP medium. Groups of 10–12 oocytes and their corresponding CCs were analysed separately. Oocytes and CCs were placed in different 1.5-mL Eppendorf tubes, snap frozen in liquid nitrogen and stored at −80°C until analysis. Poly(A) RNA was prepared as described previously (Bermejo-Álvarez *et al.* 2010; Arias-Alvarez *et al.* 2013*a*) using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech) according to the manufacturer's instructions with minor modifications. The reverse transcription (RT) reaction (Bioline) was performed using poly(T) primer, random primers and Moloney murine leukaemia virus reverse transcriptase enzyme (MMLV High-performance Reverse Transcriptase; Epicentre Biotechnologies) in a total volume of 40 µL to produce cDNA. First, tubes were heated at 70°C for 5 min to denature the secondary RNA structure and then the RT was completed with the addition of 100 units Superscript RT enzyme MMLV. The samples were subsequently incubated at 42°C for 60 min to allow RT of RNA, followed by incubation at 70°C for 10 min to denature the RT enzyme.

Quantitative real-time polymerase chain reaction in COCs

The mRNA transcripts were quantified using real-time quantitative RT–polymerase chain reaction (qRT-PCR). Experiments were conducted to determine levels of each transcript relative to that of the housekeeping gene *H2afz* (*Histone family member z*). All primers were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primersblast/) to span exon–exon boundaries when possible. All qPCR reactions were performed in duplicate using a Rotorgene 6000 RealTime Cycler (Corbett Research) after addition of 2 µL sample to the PCR mix (GoTaq qPCR Master Mix; Promega) containing specific primers to amplify the housekeeping gene, *Akt1*, *Tp53*, *Casp3*, *Sod2*, *G6pd* and *Gapdh*. *Gja1* and *Ptgs2* were quantified only in CCs because their expression is related to cell–cell communication and cumulus expansion. According to Feuerstein *et al.* (2007) and Shao *et al.* (2015), the quantification of such genes can be used as a method to evaluate the quality of granulosa cells and indirectly as a biomarker of oocyte quality. Primer sequences and the approximate size of the amplified fragments for all transcripts are given in Table 1. Cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for 10 s and 10 s of fluorescence acquisition. Each pair of primers was optimised to achieve efficiencies close to 1, and the comparative cycle threshold (C_T) method was then used to quantify expression levels as described by Schmittgen and Livak (2008). To avoid primer dimer artefacts, fluorescence was acquired in each cycle at a temperature higher than the melting temperature of primer dimers (specific for each product; 76–86°C). Then, the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background was determined for each sample. Quantification was normalised against that of the endogenous control *H2afz*. According to the comparative C_T method, the ΔC_T value was determined by subtracting the *H2afz* C_T value for each sample from each gene C_T value of the sample. Calculation of $^{\Delta\Delta}C_T$ involved using the highest sample ΔC_T value as an

arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

Confocal laser scanning microscopy in COCs

Mitochondrial staining

In all, 66 oocytes were prepared to observe the active mitochondrial migration pattern before ($n = 20$) and after ($n = 46$) the IVM period. In addition, 27 *in vivo*-matured oocytes were used to verify the mitochondrial migration pattern.

For all oocytes, CCs were mechanically removed by gentle repeated pipetting in PBS + 1 mg mL^{-1} PVP. Then, denuded oocytes were washed with PBS and incubated with 200 μM MitoTracker Orange probes (Invitrogen) at 38°C for 30 min. The MitoTracker Orange probe passively diffuses across the plasma membrane and enters live cells, where it is oxidised to the corresponding fluorescent mitochondrion-selective probe and accumulated in active mitochondria. After incubation, oocytes were fixed using 4% paraformaldehyde solution and washed with PBS. The oocytes were then stained with 1 μg mL^{-1} Hoechst 33342 dye to assess nuclear morphology related to oocyte maturation. Finally, oocytes were mounted on glass slides and observed under a laser scanning confocal microscope (Leica TCS SP2), using 546- and 351/364-nm excitation lasers to visualise mitochondria and chromosomes respectively. The format, laser, gain and offset were kept constant for every sample. The mitochondrial migration pattern was observed and classified at ×40 magnification under mineral oil. Nuclear morphology was categorised as GV, MI and MII.

Cumulus cell apoptosis assessed by terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling

To evaluate apoptosis in CCs, 37 COCs ($n = 14$, 13 and 10 from immature, IVM and *in vivo*-matured oocytes respectively) were used. COCs were washed in PBS supplemented with 1 mg mL^{-1} PVP and fixed in 4% paraformaldehyde solution for 1 h at room temperature. The COCs were pretreated with 20 mg mL^{-1} proteinase K working solution for 1 h in a humidified dark chamber at 37°C. Strand breaks of DNA occurring during the cell apoptosis process were detected using terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL; *In Situ* Cell Death Detection Kit, POD; Roche Diagnostics) with a protocol adapted from Arias-Álvarez *et al.* (2009). Positive control sections were treated with DNAse I (Roche Diagnostics) for 10 min at room temperature in a humidified chamber before incubation with the TUNEL reaction. As a negative control, samples were incubated with the label solution of the TUNEL reaction mixture without the enzymatic solution. To avoid RNA interference, COCs were treated with RNAses before staining. Finally COCs were counterstained with 0.25 μg mL^{-1} propidium iodide for 15 min and mounted between a coverslip and glass slide in mounting solution (ProLong Gold Antifade Reagent; Invitrogen). Samples were observed under a laser scanning confocal microscope (Leica TCS SP2) using a 488-nm excitation laser to visualise TUNEL-positive cells and a 546-nm excitation laser to assess red fluorescence. The format, laser, gain and offset were kept constant for all samples. Images were analysed using ImageJ software (http://rsbweb.nih.gov/ij/). The apoptosis index was calculated as the green area divided by the red area × 100.

Assessment of the developmental competence of oocytes

The developmental competence of oocyte was evaluated by IVF and *in vitro* culture (IVC) procedures as described previously (Zeng *et al.* 1999; Viudes-de-Castro *et al.* 2005) with some modifications described below. In all, 350 IVM and *in vivo*-matured oocytes were used in two replicates.

IVF was performed using a pool of heterospermic fresh semen collected using an artificial vagina from five adult bucks. The motility of ejaculated spermatozoa was >85%. The sperm samples were diluted 1 : 50 in fertilisation medium (Tyrode's medium with sodium bicarbonate (2 mM), sodium lactate (36 mM), sodium pyruvate (1 mM), 0.01% BSA and heparin (10 μg mL^{-1})), washed with fertilisation medium and centrifuged twice at 1000*g* for 5 min at room temperature each time. The spermatozoa were then subjected to a swim-up procedure in 2 mL Tyrode's medium for 20 min under an atmosphere of 5% CO_2 in air with maximum humidity at 38°C. The spermatozoa recovered from the supernatant were incubated for a further 6 h under the same conditions. Then, IVM and *in vivo*-matured oocytes were coincubated with spermatozoa at a final concentration of 1×10^6 spermatozoa mL^{-1} for 4 h at 38°C in four-well dishes containing 50 COCs in 500 mL Tyrode's medium per well.

After the coincubation, presumptive zygotes were denuded by gentle pipetting, washed four times in PBS and cultured in TCM-199 supplemented with 20% (v/v) fetal calf serum (FCS), as described previously (Arias-Álvarez *et al.* 2010). Then, groups of 25 zygotes were placed in 25-μL droplets of culture medium overlaid with mineral oil and incubated for 60 h under an atmosphere of 5% CO_2 in air with maximum humidity at 38.5°C. Differences in oocyte developmental competence between *in vivo*-matured and IVM oocytes were scored according to embryo development at 12, 36 and 60 h post-insemination (hpi). Embryos were classified on the basis of conventional morphological criteria according to their developmental stage following the guidelines of the International Embryo Transfer Society (www.iets.org). The number of 2- to 4-cell embryos, 8- to 16-cell embryos, early morulae, morulae and early blastocysts was expressed as a percentage of the total number of presumptive zygotes.

Statistical analysis

Data were analysed using SPSS Version 19 (IBM SPSS Statistics). The Shapiro–Wilks test was used to assess normality and homogeneity of variance. Comparisons of the apoptosis rate and mRNA transcript expression between groups were made using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test or a Kruskal–Wallis test for non-parametric samples. Nuclear maturation categories, mitochondrial patterns and *in vitro* early embryo development were compared using a Chi-squared test. All data are expressed as the mean ± s.e.m. and two-tailed $P < 0.05$ was considered significant.

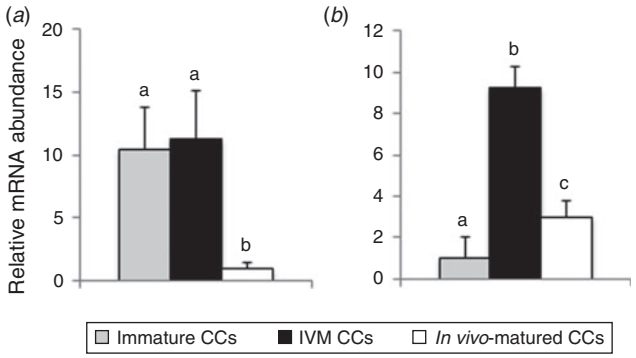

Fig. 1. Relative mRNA abundance of (*a*) gap junction protein, alpha 1, 43 kDa (*Gja1*) and (*b*) prostaglandin-endoperoxide synthase 2 (*Ptgs2*) in cumulus cells (CCs) of germinal vesicle (immature), IVM and *in vivo*-matured oocytes. Data are the mean ± s.e.m. Columns with different letters differ significantly ($P < 0.05$).

Results

Transcript abundance of quality-related genes in COCs

The relative abundance of genes related to cumulus expansion, namely *Gja1* and *Ptgs2*, in CCs is shown in Fig. 1. No significant differences were observed in the abundance of *Gja1* transcripts before and after IVM. However, *Gja1* expression was significantly lower in CCs from *in vivo*-matured oocytes (Fig. 1*a*; $P < 0.05$). Transcripts of *Ptgs2* were upregulated after *in vitro* and *in vivo* oocyte maturation ($P < 0.05$); nevertheless, *Ptgs2* mRNA expression was significantly higher in CCs from COCs matured *in vitro* than from those matured *in vivo* (Fig. 1*b*; $P < 0.05$).

The expression of the other transcripts evaluated in both oocytes and CCs are shown in Fig. 2. In oocytes, *Akt*, *Tp53*, *Casp3*, *Sod2* and *G6pd* poly(A) mRNA content was significantly downregulated after *in vivo* maturation compared with IVM and GV (immature) oocytes ($P < 0.05$). Only *Sod2*

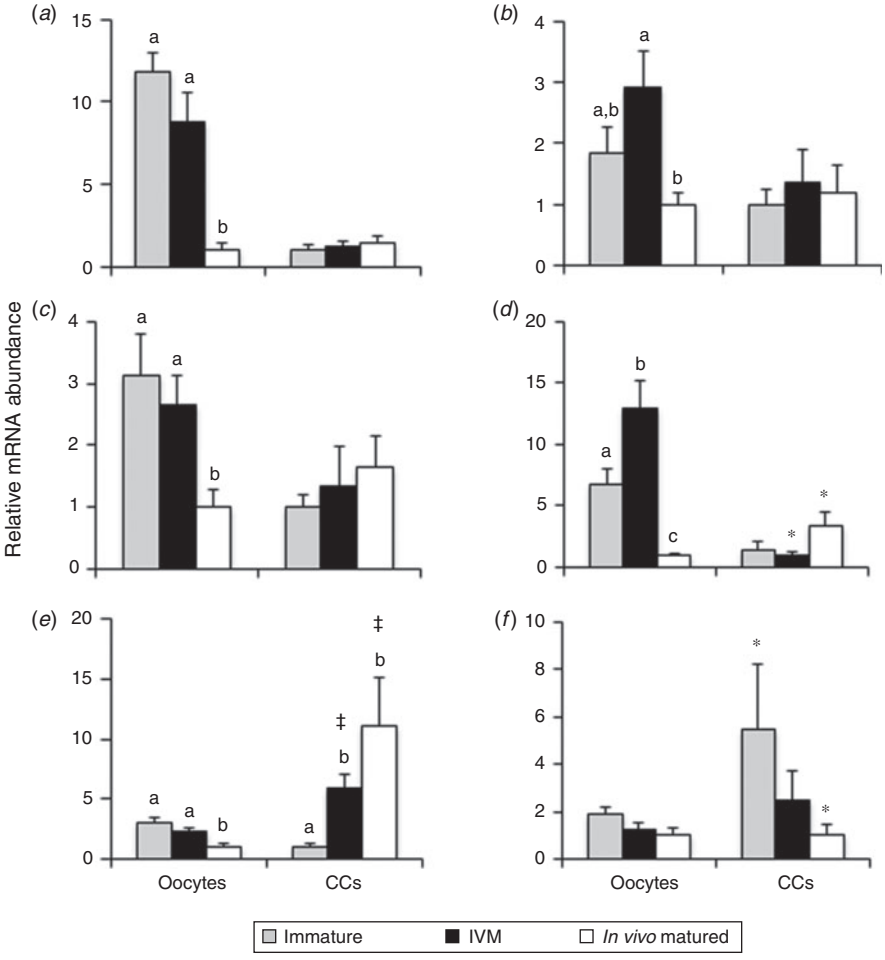

Fig. 2. Relative mRNA abundance of genes in immature, IVM and *in vivo*-matured oocytes and cumulus cells (CCs). (*a*) V-Akt murine thymoma viral oncogene homologue 1 (*Akt1*), (*b*) tumour protein 53 (*Tp53*), (*c*) caspase 3, apoptosis-related cysteine protease (*Casp3*), (*d*) superoxide dismutase 2, mitochondrial (*Sod2*), (*e*) glucose-6-phosphate dehydrogenase (*G6pd*) and (*f*) glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Data are the mean ± s.e.m. Columns with different letters differ significantly. Data with * differs ($P = 0.06$) and ‡ differs ($P = 0.1$).

poly(A) mRNA content was significantly increased in oocytes after IVM compared with GV stage oocytes ($P < 0.05$). However, no differences in the gene expression of *Akt*, *Tp53* and *Casp3* were found in CCs among the experimental groups. Although *Sod2* expression tended to be upregulated in CCs from *in vivo*-matured COCs compared with IVM COCs ($P = 0.06$), *Sod2* mRNA expression was similar before and after oocyte maturation. *G6pd* transcripts were upregulated after oocyte maturation in CCs ($P < 0.05$) and tended to be higher after *in vivo* maturation ($P = 0.1$). The mRNA abundance of *Gapdh* tended to be lower in CCs from *in vivo*-matured COCs compared with immature COCs ($P = 0.06$), with values in IVM CCs being intermediate between these two groups.

Oocyte maturation and mitochondrial distribution patterns

As shown in Fig. 3, the mitochondrial migration pattern found was classified as either: (1) diffused homogeneous (DH), with fluorescence dispersed throughout the entire cytoplasm; (2) diffused clustered (DC), with aggregates of fluorescence dispersed throughout the cytoplasm; (3) migrated homogeneous (MH), with high fluorescence in the cortical area and moderate through the cytoplasm; and (4) migrated clustered (MC), with higher fluorescence in cortical areas with aggregates. Before IVM, 100% of oocytes exhibited GV nuclear morphology. After IVM, 63% of oocytes were at the MII stage ($n = 29$), 28.3% were at the MI stage ($n = 13$) and 8.7% were at the GV stage ($n = 4$). All *in vivo*-matured oocytes exhibited the MII configuration.

Fig. 4 shows that the immature oocytes mainly exhibited a DH mitochondrial distribution ($P < 0.001$), whereas the most prevalent mitochondrial pattern in *in vivo*-matured oocytes was the MH pattern ($P < 0.001$). In the IVM group, the mitochondrial migration pattern most frequently observed was MC rather than MH ($P < 0.05$). The rest of mitochondrial patterns defined in this group (DH, DC) showed intermediate values. Comparing experimental groups, MH distribution was significantly higher in *in vivo*-matured MII oocytes than in IVM or GV (immature) oocytes ($P < 0.001$). The MC pattern was significantly more frequent in IVM oocytes than in the other experimental groups ($P < 0.05$). The DC pattern was also more frequent in IVM than *in vivo*-matured oocytes ($P < 0.05$), with immature oocytes having intermediate values. The percentage of oocytes with the DH pattern of mitochondrial distribution increased significantly in the immature oocyte group compared with the *in vivo*-matured group ($P < 0.001$). After IVM, oocytes tended to have

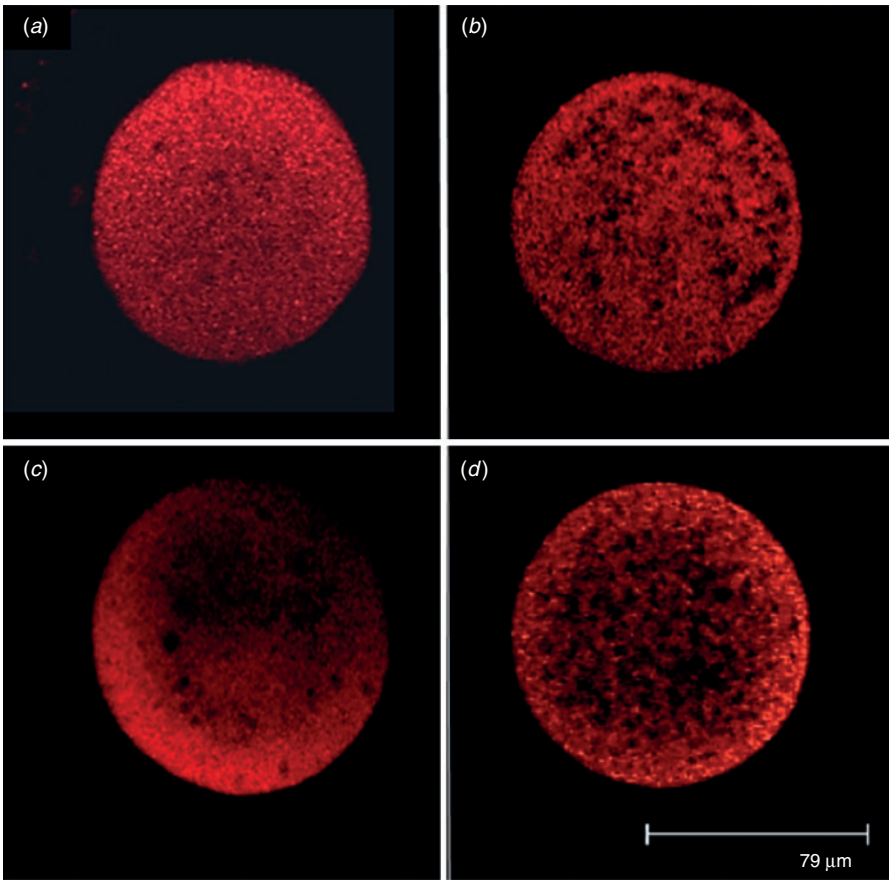

Fig. 3. Mitochondrial relocation in rabbit oocytes. Mitochondrial migration patterns were classified as (*a*) diffused homogeneous, (*b*) diffused clustered, (*c*) migrated homogeneous or (*d*) migrated clustered (MC).

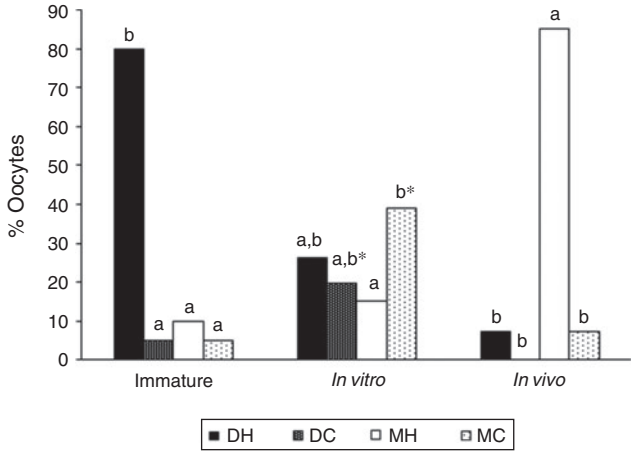

Fig. 4. Percentage of germinal vesicle (immature), IVM and *in vivo*-matured oocytes exhibiting the different mitochondrial distribution patterns. Data are the mean ± s.e.m. Within each group, columns with different letters differ significantly. Data with * differs ($P = 0.1$). DH, diffused homogeneous; DC, diffused clustered; MH, migrated homogeneous; MC, migrated clustered.

higher values of the DH pattern than *in vivo*-matured oocytes ($P = 0.1$), but there was no significant difference between the IVM and immature groups.

Apoptosis of CCs

The percentage of apoptosis was significantly lower in COCs matured *in vivo* compared with those matured *in vitro* or the immature group ($6.0 \pm 1.0\%$ vs $10.9 \pm 1.7\%$ and $11.0 \pm 2.0\%$ respectively; $P < 0.05$; Fig. 5). The rate of apoptosis was similar in CCs from immature and IVM oocytes.

Developmental competence of oocytes

Cumulus expansion just before coincubation with spermatozoa is shown in Fig. 6. Morphological differences can be seen between IVM and *in vivo*-matured groups. Ovulated oocytes seem to have a better pattern of CC expansion.

As indicated in Table 2, the kinetics of early embryo development were affected by the origin of the embryos (*in vitro* or *in vivo*). The cleavage rate after IVF increased significantly ($P < 0.01$) in embryos derived from ovulated oocytes compared with those derived from IVM oocytes.

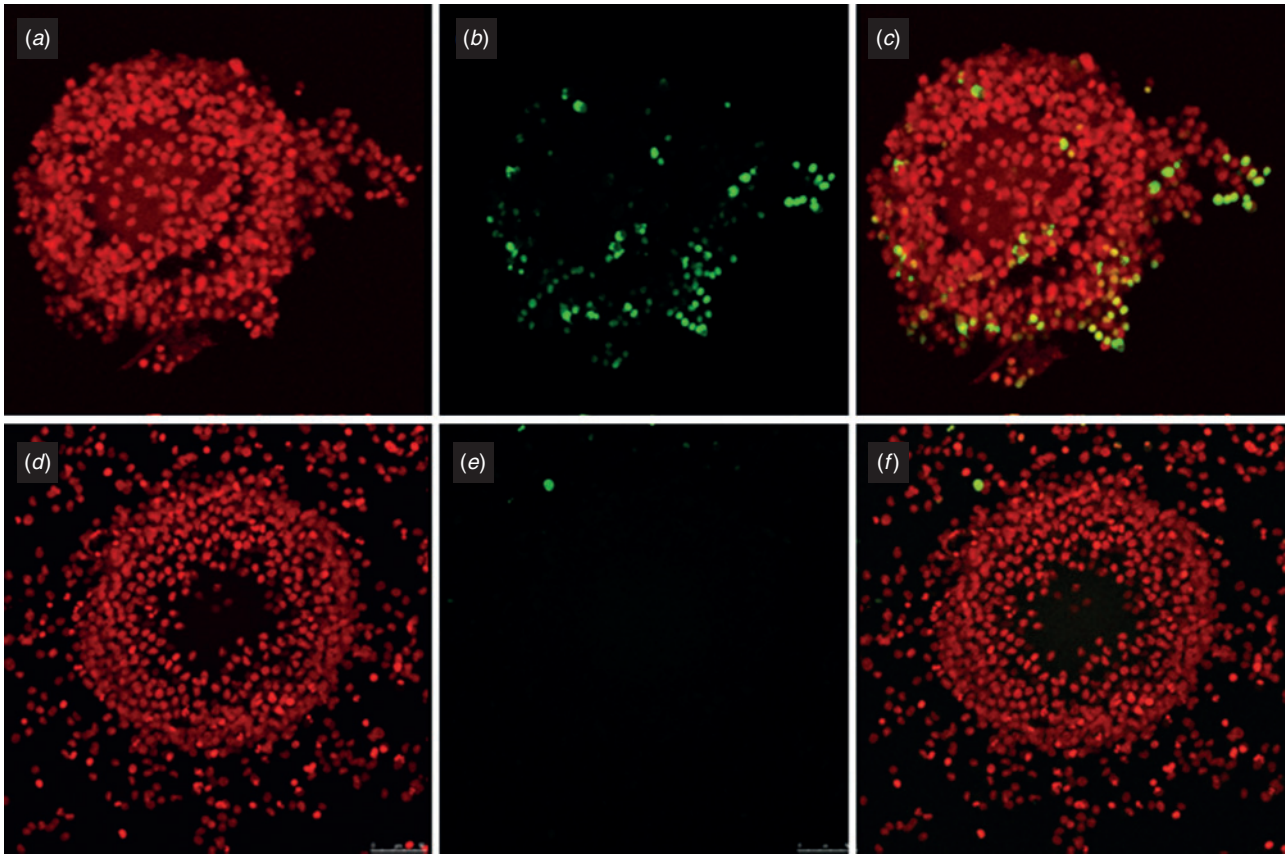

Fig. 5. Cumulus cell apoptosis assessed by the terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) assay in rabbit cumulus–oocyte complexes (COCs) after (*a–c*) IVM and (*d–f*) *in vivo* maturation. (*a*, *d*) Maximal projection of a complete COC showing the nuclei of cumulus cells stained with propidium iodide (red). (*b*, *e*) Maximal projection of a complete COC showing TUNEL-positive cells considered as apoptotic (green). (*c*, *f*) Maximal projection of a complete COC overlapping both fluorochromes. Images were taken at an original magnification of ×40.

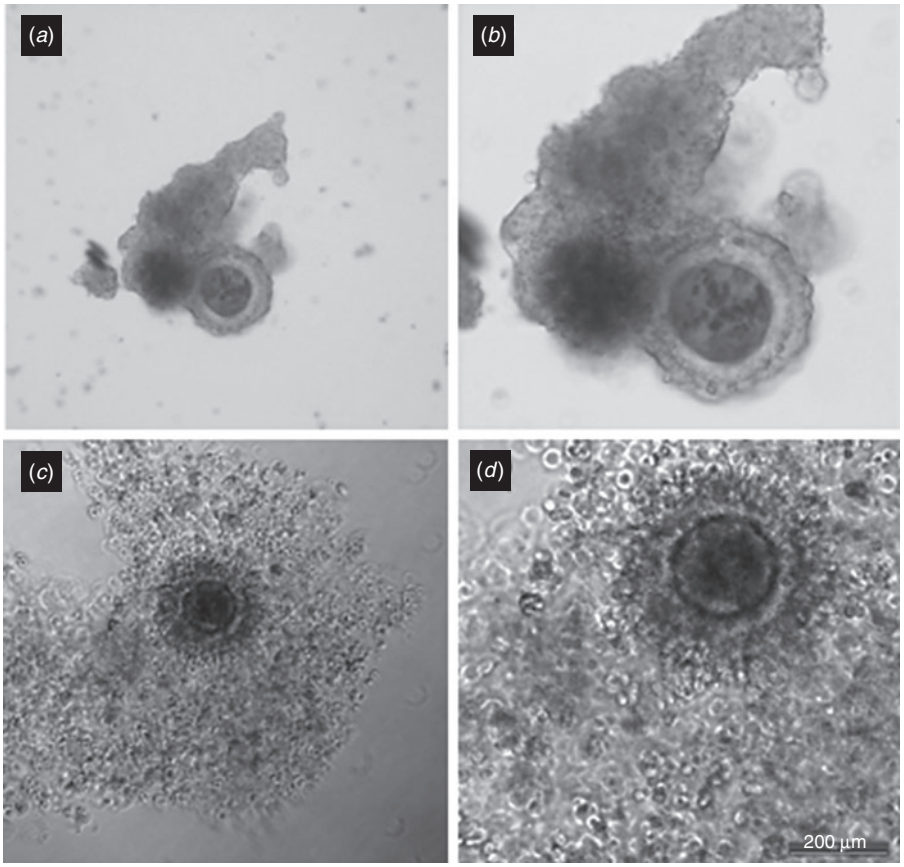

Fig. 6. Cumulus–oocyte complexes after (*a*, *b*) IVM or (*c*, *d*) *in vivo* maturation. Scale bar = 200 µm.

Table 2. Early development of rabbit embryos derived from *in vivo*-matured and IVM oocytes
Within columns, values with different letters differ significantly ($P < 0.01$). Values with * differ ($P = 0.08$)

	Presumptive zygotes	Total cleaved	12 h	36 h			60 h			
			2- to 4-cells	2- to 4-cells	8- to 16-cells	Early morulae	2- to 4-cells	8- to 16-cells	Morulae	Early blastocysts
In vitro-derived oocytes										
n	184	44[a]	33[a]	39*	5[a]	0[a]	36	1[a]	6[a]	1
%		23.9	17.9	21.2	2.7	0.0	19.5	0.5	3.3	0.5
In vivo-recovered oocytes										
n	160	79[b]	47[b]	47*	26[b]	6[b]	35	22[b]	15[b]	2
%		49.3	29.4	29.4	16.2	3.7	21.8	13.8	9.4	1.3

The percentage of 2- to 4-cell embryos at 12 hpi was significantly higher in the group of *in vivo*-derived oocytes compared with the *in vitro* group ($P < 0.01$). Similarly, the percentage of all categorised embryos at 36 and at 60 hpi was significantly greater in the group derived from *in vivo*-matured compared with IVM oocytes ($P < 0.01$), except for the early blastocyst rate.

Discussion

In vivo-matured COCs seem to be an adequate model in which to identify biomarkers to evaluate the developmental competence of oocytes and to understand the molecular mechanisms underlying the communication between oocytes and CCs. The findings of the present study corroborate that the gene expression profile of *in vivo*-matured rabbit COCs is different from that of IVM COCs. These changes in gene expression are clearly accompanied by stage-specific cytoplasmic remodelling of mitochondria, a lower rate of apoptosis of *in vivo*-matured CCs and better early embryo development, indicating that oocyte quality is compromised during IVM.

Cumulus expansion is positively linked to oocyte quality, which is widely used to evaluate the maturity of COCs. GJA1, or

connexin 43, has been proposed as a major mediator of cell–cell communication via gap junctions (Gittens and Kidder 2005) and has been identified as a non-invasive biomarker for the fertilisation potential of human oocytes (Feuerstein *et al.* 2007; Hasegawa *et al.* 2007). The findings of the present study show that *Gja1* expression was significantly lower CCs surrounding *in vivo*-matured MII oocytes than in CCs enclosing immature or IVM oocytes, supporting previous results obtained in the bovine (Calder *et al.* 2003), canine (Cho *et al.* 2016) and in humans (Li *et al.* 2015). The reduced expression of *GJA1* in CCs after oocyte maturation is beneficial (Edry *et al.* 2006) because it is related to cumulus expansion and decreased diffusion of cAMP and cGMP from the CCs to the oocyte, which is followed by the end of meiotic arrest and extrusion of the first polar body (Shao *et al.* 2015).

Conversely, PTGS2 is the key enzyme in prostaglandin E biosynthesis and is also involved in regulation of CC expansion during the ovulatory process in mice (Davis *et al.* 1999; Jang *et al.* 2015). In the present study, *Ptgs2* mRNA transcripts were upregulated in CCs after oocyte maturation, as described previously for cattle (Nuttinck *et al.* 2002), mice (Shao *et al.* 2015) and humans (Feuerstein *et al.* 2007; Wathlet *et al.* 2011). The abundance of *PTGS2* in CCs is positively correlated with oocyte competence in other species, including cattle (Assidi *et al.* 2008), pigs (Kang *et al.* 2012) and humans (McKenzie *et al.* 2004). However, in the present work, *Ptgs2* mRNA expression was significantly lower in CCs from *in vivo*-matured rabbit COCs, which exhibited better CC expansion and higher developmental competence after IVF. In contrast with these results, previous reports in the bovine (Tesfaye *et al.* 2009) and humans (Ouandaogo *et al.* 2012) have found that *PTGS2* is lower in CCs from oocytes matured *in vitro* compared with those matured *in vivo*. The reason for this discrepancy could be associated with the ovarian stimulation protocols used, which could interfere in the endocrine and cytokine environment and induce changes in the gene expression in CCs, as reported previously in mice (Foster *et al.* 2010), rats (Agca *et al.* 2013) and women (Smitz *et al.* 2007; Adriaenssens *et al.* 2010; Wathlet *et al.* 2011). In fact, *PTGS2* mRNA decreases after human chorionic gonadotrophin administration in equine granulosa cells (Boerboom and Sirois 1998). Taking these observations into account, the present study cannot confirm *Ptgs2* as a candidate biomarker of oocyte competence in the rabbit.

Although in rabbits the chromosomes are tightly condensed in oocytes enclosed in antral follicles ≥1 mm in diameter (Jelíanková *et al.* 1994), RNA transcription is still active and the oocyte fully meiotic competent (Wang *et al.* 2009). During meiotic maturation, a global decrease in transcription levels of the accumulated maternal mRNA is expected (Bachvarova 1985; Kohata *et al.* 2013), because translated transcripts undergo deadenylation and selective degradation (Paynton and Bachvarova 1994). In the present study, most of the mRNA transcripts associated with cell cycle checkpoint, apoptosis and metabolic pathways were downregulated in *in vivo*-matured MII oocytes compared with immature GV and IVM oocytes. Dysregulation in either gene transcription or post-transcriptional modification of genes in IVM oocytes would result in incorrect temporal utilisation of those transcripts, which may culminate in reduced developmental competence (Zheng *et al.* 2005*a*; Jones *et al.* 2008). The serine/threonine kinase Akt contributes to the regulation of the meiotic cell cycle in mammalian oocytes (Kimura *et al.* 2007). Phosphorylation and activation of Akt during oocyte maturation precedes GV breakdown in mouse oocytes (Kalous *et al.* 2006). The results of the present study regarding *Akt1* poly(A) transcripts are in agreement with this finding and with previous studies in which higher *AKT1* expression during IVM confirms that culture conditions can affect the molecular mechanisms controlling nuclear and cytoplasmic maturation (Cecconi *et al.* 2010). *TP53* also has a crucial role in cell cycle regulation because it participates in the G_1/S phase checkpoint and therefore in cell cycle arrest. *TP53* is involved in DNA repair and regulates the expression of target genes, such as *CASP3*, leading to cell death through apoptosis when DNA damage occurs (Zheng *et al.* 2005*b*). The upregulation of *Tp53* and *Casp3* in IVM oocytes in the present study supports dysfunction in cell cycle regulation and suggests an increase in DNA repair mechanisms, leading to cell cycle arrest and apoptosis. *SOD2* is involved in the oxidative response and mitochondrial activity because it encodes a mitochondrial protein that catalyses the dismutation of superoxide into H_2O_2. In the present study, we found a higher poly(A) mRNA abundance of *Sod2* in IVM oocytes compared with those matured *in vivo*. This finding indicates higher oxidative stress in oocytes during IVM (Maître *et al.* 1993; Guérin *et al.* 2001) and may suggest a readenylation process involved in the regulation of the expression of this transcript, as described previously in mice and cattle (Brevini-Gandolfi *et al.* 1999). However, in CCs, opposite findings regarding gene abundance were made: *Sod2* mRNA was higher in CCs from *in vivo*-matured compared with IVM oocytes. CCs with higher *SOD2* activity may effectively protect COCs against apoptosis caused by the generation of ROS. In fact, increased SOD2 activity in the follicular fluid has been associated with better oocyte quality in humans (Kably Ambe *et al.* 2004). This finding is in agreement with the lower apoptosis rates determined using the TUNEL assay in CCs from IVM COCs compared with those matured *in vivo*. In fact, it has been reported that the incidence of apoptosis in CCs during IVM reduces oocyte quality and induces the failure of meiotic maturation in gilts (Yuan *et al.* 2008), as well as impaired oocyte developmental competence in humans (Lee *et al.* 2001).

Glucose metabolism through the pentose phosphate pathway (PPP) is a primary factor for the progression of nuclear and cytoplasmic maturation through the provision of substrates for purine synthesis, such as phosphoribosylpyrophosphate (PRPP) and redox homeostasis (reduced nicotinamide adenine dinucleotide phosphate (NADPH) through reduction of glutathione (for a review, see Sutton-McDowall *et al.* 2010). This contributes to certain level of protection against oxidative stress-induced apoptosis in the oocyte (Combelles *et al.* 2009). In the present study, the gene encoding the enzyme that catalyses the first and irreversible step of the PPP pathway (*G6pd*) was upregulated in CCs after oocyte maturation and tended to be higher in *in vivo*-matured CCs, implying a possible increase in the PPP pathway and higher redox protection (Downs *et al.* 1998; Kang *et al.* 2012). Moreover, *G6pd* was downregulated in oocytes after *in vivo* maturation compared with immature and IVM oocytes.

Ovine (Wang *et al.* 2012) and rat (Tsutsumi *et al.* 1992) oocytes with decreased G6PD activity have been shown to have higher developmental competence as well. This finding corresponds to the lower potential PPP activity in MII oocytes reported in mice (Urner and Sakkas 1999; Dumollard *et al.* 2007).

Several authors have suggested that the glucose within COCs is essentially destined for the production of pyruvate and lactate, because both are the main preferred substrates for the oocyte. GAPDH catalyses the sixth step of anaerobic glycolysis, converting NAD^+ to NADH and thus generating reducing power. Increased abundance of the *GAPDH* transcript in both oocytes and CCs has been associated a higher oocyte competence after IVM in cattle (Bermejo-Álvarez *et al.* 2010; Gendelman and Roth 2012). Paradoxically, in the present study we found that *Gapdh* mRNA transcripts decreased slightly in CCs after *in vivo* maturation compared with CCs from immature GV oocytes. These differences between experimental groups dissuaded us from using this gene as an internal control in the quantitative analysis of gene expression, in contrast with previous reports in dogs (Cho *et al.* 2016), mice (Shao *et al.* 2015) and humans (Ouandaogo *et al.* 2012), but support previous findings in rabbit embryos (Arias-Álvarez *et al.* 2013*a*, 2013*b*), mouse oocytes (Jeong *et al.* 2005; Cui *et al.* 2007) and bovine oocytes (Bermejo-Álvarez *et al.* 2010; Adona *et al.* 2016). These controversial results indicate that the expression of *GAPDH* could differ among species and this deserves further investigation.

With regard to the events accompanying cytoplasmic maturation, the present study has established, for the first time, mitochondrial distribution patterns in rabbit oocytes. The oocytes exhibited changes in mitochondrial organisation, ranging from a fine uniform distribution throughout the cytoplasm in immature GV oocytes to cortical distribution as maturation progressed with both IVM and *in vivo* maturation. The percentage of oocytes with migrated mitochondria was significantly lower after IVM than *in vivo* maturation. Furthermore, in IVM oocytes, mitochondria formed aggregates, whereas most *in vivo*-matured MII oocytes had a cortical and homogeneous pattern of mitochondrial distribution. Although mitochondrial distribution patterns have been described in different species, including mice (Yu *et al.* 2010; Nazmara *et al.* 2014), pigs (Sun *et al.* 2001), cattle (Zhuang *et al.* 2012), horses (Torner *et al.* 2007), cats (De los Reyes *et al.* 2011) and humans (Guérin *et al.* 2001; Liu *et al.* 2010), it has been reported that homogeneous mitochondrial distribution is correlated with lower oxidative activity and with minor levels of CC apoptosis, whereas a clustered distribution is correlated with the opposite (Torner *et al.* 2004). Therefore, the high percentage of clustered mitochondrial patterns in IVM oocytes observed in the present study could contribute to the lower developmental potential of these oocytes compared with oocytes matured *in vivo*.

The findings of the present study are in agreement with the different kinetics of early cleavage divisions observed in embryos derived from *in vivo*-matured compared with IVM oocytes. Previous studies have demonstrated that the timing of the first cleavage divisions (Lonergan *et al.* 1999), activation of the embryonic genome at the 8- to 16-cell stage in rabbits and compaction of the morula determine the quality of embryos (Rizos *et al.* 2008). Such events, especially those that sustain early embryo development until activation of the embryonic genome, are directly related to the intrinsic quality of the oocyte (Lonergan *et al.* 1999). In the present study, embryos derived from ovulated oocytes showed faster developmental speed and a higher percentage of presumptive zygotes that cleaved and developed to the morula stage, which provides clear evidences of the higher quality of these embryos (Gutiérrez-Adán *et al.* 2004; Rizos *et al.* 2008). These findings corroborate the results for the mRNA transcripts, mitochondrial distribution and rate of apoptosis of CCs in rabbit COCs in the present study.

Conclusions

The present study provides an original description of the cellular and molecular events during oocyte maturation in the rabbit model. Differences in the gene expression profile related to cumulus expansion, cell cycle regulation, apoptosis and oxidative stress; mitochondrial reallocation; and the cumulus apoptotic index between *in vivo*-matured and IVM rabbit COCs support the idea that the functions mediated by these genes could be compromised during IVM, producing MII oocytes of suboptimal quality. General increased mRNA poly(A) abundance in IVM oocytes suggests a failure to undergo the normal pattern of transcript silencing expected in good-quality oocytes and could explain the poor developmental competence of IVM versus *in vivo*-matured oocytes. In fact, embryos derived from *in vivo*-matured oocytes exhibited higher developmental ability during the first cleavage divisions than those derived from IVM oocytes. Among the genes studied in CCs, we consider the expression of *Gja1* as a possible non-invasive biomarker of oocyte developmental competence in this species. The findings of the present study set the basis for further investigations to improve IVM conditions and therefore contribute to the progress of in ARTs.

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